Enhanced Anti-Tumor Activity Of an anti-5T4 Antibody-Drug Conjugate in Combination with PI3K/mTOR inhibitors or Taxanes

Boris Shor¹, Jennifer Kahler¹, Maureen Dougher¹, Jane Xu¹, Michelle Mack¹, Ed Rosfjord¹, Fang Wang¹, Eugene Melamud¹, Puja Sapra¹*.

¹Oncology Research Unit, Pfizer Worldwide Research and Development, NY, USA

*Corresponding author: Puja Sapra, PhD, Bioconjugates Discovery and Development, Oncology Research Unit, Pfizer Worldwide Research and Development, 401 North Middletown Road, Pearl River, NY, 10965. Email: puja.sapra@pfizer.com. Phone: (845) 602-3389. Fax: (845) 602-5557.

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Disclosure of Potential Conflicts of Interest

All authors are/or were full time employees of Pfizer Inc. when research was conducted and own company's stock options and/or units.
Translational Relevance

Antibody-drug conjugates (ADCs) are emerging as a promising therapeutic modality for the treatment of cancers. However, despite encouraging activity, patients treated with single agent ADCs show relapse or progression due to emergence of resistance. Combination of ADCs with other chemotherapeutic or targeted agents is being evaluated in the clinic to improve the overall response. In this study, in preclinical models, we systematically evaluated combination partner(s) for an auristatin-based antibody-conjugate (5T4-ADC) and demonstrate enhanced activity when 5T4-ADC was combined with PI3K/mTOR inhibitor or with taxanes. Our findings provide rationale to evaluate 5T4-ADC in combination with PI3K/mTOR pathway inhibitors or taxanes in the clinic. Additionally, our data suggest that the mechanistic basis of the observed synergy may be attributed to the mechanism of action of payload, i.e. auristatin. As several auristatin-based ADCs are in clinical trials, we believe our findings can be applicable to other ADCs employing auristatins.
Abstract

Purpose:

Targeted treatment of solid or liquid tumors with antibody-drug conjugates (ADCs) can lead to promising clinical benefit. The aim of the study is to investigate combination regimens of auristatin-based ADCs in preclinical models of cancer.

Experimental Design:

An auristatin-based anti-5T4 antibody conjugate (5T4-ADC) and auristatin payloads were combined with the dual PI3K/mTOR catalytic site inhibitor PF-05212384 (PF-384) or taxanes in a panel of tumor cell lines. Drug interactions in vitro were evaluated using cell viability assays, apoptosis induction, immunofluorescence, mitotic index, and immunoblotting. Breast cancer cells treated with auristatin analog or 5T4-ADC were profiled by total- and phospho-proteomics. Anti-tumor efficacy of selected combinations was evaluated in 5T4-positive human breast or lung tumor xenografts in vivo.

Results:

In vitro, auristatin-based agents displayed strong synergistic or additive activity when combined with PF-384 or taxanes respectively. Further, treatment of 5T4-ADC plus PF-384 resulted in stronger induction of apoptosis and cell-line-specific attenuation of pAKT and pGSK. Interestingly, proteomic analysis revealed unique effects of auristatins on multiple components of mRNA translation. Addition of PF-384 further amplified effects of 5T4-ADC on translational components, providing a potential mechanism of synergy between these drugs. In human tumor xenografts, dual targeting with 5T4-ADC/PF-384 or 5T4-ADC/paclitaxel produced substantially greater antitumor effects with longer average survival as compared to monotherapy treatments.

Conclusions:

Our results provide a biologic rationale for combining 5T4-ADC with either PI3K/mTOR pathway inhibitors or taxanes and suggest that mechanisms underlying the synergy may be attributed to cellular effects of the auristatin payload.
Introduction

Antibody drug conjugates (ADCs) are poised to become an important class of cancer therapeutics, as evidenced by the promising response rates when administered as single agents to chemorefractory cancer patients. However, despite significant survival benefit, most patients eventually relapse or show disease progression after treatment with single-agent ADCs (1-4). One strategy to improve clinical outcomes for ADCs is through combination with chemotherapy or molecularly targeted agents. Most of the known ADCs undergoing clinical testing contain tubulin inhibitors, including auristatins and maytansinoids. Auristatins are fully synthetic water soluble dolastatin analogs that account for over 50% of ADCs in clinical development. Mechanistically, auristatins bind the same site on tubulin as the vinca alkaloids, destabilize microtubules, block microtubule assembly and arrest cells in the G2/M phase of the cell cycle resulting in cell death (5, 6). Currently, two auristatins, monomethyl auristatin-E (MMAE) and monomethyl auristatin-F (MMAF) are being investigated in the context of ADCs, with several other auristatin analogs entering clinical development. We have previously described an ADC that targets 5T4, an oncofetal antigen expressed on tumor-initiating cells (TIC) (7). The 5T4-mcMMAF (5T4-ADC) is comprised of a humanized anti-5T4 A1 antibody linked to the MMAF via a noncleavable maleimidocaproyl (mc) linker and thus, the ADC’s mechanism of action is mechanistically linked to the biological activity of the auristatin moiety that is liberated upon internalization of the ADC into the cancer cell.

The PI3K/mTOR pathway plays an essential role in oncogenesis, and is implicated in the emergence of resistance to several therapeutic drugs in clinical settings including erlotinib, gefitinib or trastuzumab in patients with HER2-positive solid cancers (8-11). Accordingly, agents that normalize PI3K/mTOR pathway activity including direct inhibitors of PI3K (GDC-
0941), AKT (MK-2206) or mTOR (everolimus) have shown promising activity in trastuzumab-resistant patients when combined with trastuzumab both in preclinical and clinical investigations (12-15). PF-05212384 (PF-384) is a pan-class I isoform PI3K and mTORC1/2 inhibitor, which is in the early stages of clinical development for solid tumor indications (16, 17). Unlike PI3K pathway inhibitors, taxanes are cytotoxic agents that are used as a first or second line therapy for many malignancies including breast, ovarian or lung cancers (18, 19). Paclitaxel (PTX) and docetaxel are the two most common taxanes, which arrest mitotic division by binding to β-tubulin and stabilizing pre-existing microtubules (20, 21). With evidence of single-agent activity in the clinical setting, taxanes have been evaluated extensively, but mostly empirically in combination with other chemotherapy agents including platinum compounds, anthracyclines or radiation (22).

This report identifies a novel therapeutic strategy to increase efficacy of 5T4-ADC by combining it with PF-384 or PTX and suggests that a similar approach can be applied to the broad class of auristatin-based drugs. We aimed to evaluate cellular responses of a subset of 5T4-positive tumor cells to co-treatment with 5T4-ADC/PF-384 or 5T4-ADC/PTX. By applying global proteomics approaches, we identify auristatin-specific pathway perturbations that could give insight into the mechanisms of synergy. Our results reveal modest but selective changes in cellular mRNA translation machinery with 5T4-ADC or auristatin, an effect which is greatly amplified by the addition of PF-384. When tested in xenograft models of breast and lung cancer, co-targeting with 5T4-ADC/PF-384 or 5T4-ADC/PTX combinations results in enhanced antitumor activity and thus may have a potential to generate more durable responses in 5T4-positive patients in the clinic.
Materials and Methods

Cell Lines and reagents

Human tumor cell lines NCI-H1975, Calu-6, NCI-H358, HCC2429, MDA-MB-468, MDA-MB-231, CAOV-3, TOV-112D, OV-90, OVCAR-3, SKOV-3, HT-29, NCI-N87, Raji, and Ramos were purchased from the American Type Culture Collection (ATCC). MDAMB361-DYT2 cells were obtained from Dr. D. Yang (Georgetown University, Washington, D.C.). Cell lines were authenticated annually by short-tandem repeat analysis (Promega STR profiling service) and routinely tested for mycoplasma contamination (ATCC). MDAMB435/5T4 are cells stably transfected with human 5T4 were described previously (23). The 37622A1 NSCLC patient-derived xenograft (PDX), and the establishment and characterization of primary serum-free culture TUM622 from 37622A1, were described (24). Each cell line was cultured in its standard medium as recommended by ATCC. For in vitro studies, chemotherapeutic drugs were obtained from Sigma-Aldrich. PF-05212384 (PKI-587), MMAF-Ome and auristatin 101 were obtained from Pfizer WWMC. Preparation of 5T4-ADC (A1mcMMAF) was described previously.

Synergy Assays

The effects of drug combinations were evaluated using Chou-Talalay median effect analysis (25). Cells were treated with each drug alone and in combination in two independent 96-well plates in a diagonal matrix format, and proliferation was measured by using a CellTiter Glo kit (Promega). Results were expressed as surviving fractions (fraction affected, Fa), based on the measured luminescence counts of treated samples, compared to that of untreated controls. 7 diagonals representing various dose-effect curves with fixed drug ratios were used to measure
the combination indexes (CI) for each of the combinations with CalcuSyn software (Biosoft, Ferguson, MO). In each experiment, CI indexes at ED50 levels were averaged for the three dose effect curves that had 7 to 8 data points. The CIs from two to three independent experiments were averaged to generate a single number shown in Figs. 1A and 4A. The Chou-Talalay method was employed to calculate combination index (CI), with the CI values of $< 0.9$ considered as evidence of synergy; $0.9 - 1.1$, additive effects; CI $> 1.1$, antagonism (25).

### Western Blotting

Equal amounts of proteins were subjected to immunoblotting analysis using NuPAGE electrophoresis system (Life Technologies). The primary antibodies for P-AKT (S473, S308), AKT, P-GSK(S21/9), GSK, P-eIF4G1 (S1108), eIF4G1, eIF4G2, P-eIF4B (S422), eIF4B, P-eIF2α (S51), eIF2α, eIF3A, eP-eEF2 (T56), eEF2, 4E-BP1 (T37/46), 4E-BP1, P-H3, cPARP, GAPDH, P-Aurora A (T288) /B (T232)/C (T198), total Aurora B and secondary antibodies were obtained from Cell Signaling Technology. Antibodies for total 5T4 were from Abcam (EPR5530/ab129058) or described previously (24).

### Xenograft efficacy studies

Female athymic nu/nu mice (18-23g) were obtained from Charles River Laboratories, Wilmington, MA. Mice were injected with tumor cells subcutaneously and animals with staged tumors were administered intravenously with saline (vehicle), 5T4-ADC, PF-384, PTX, or combinations 5T4-ADC plus PF-384, 5T4-ADC plus PTX. ADCs were administered based on mAb protein content at 2 or 3 mg Ab/kg on a Q4D (every 4 day) schedule, with 8 to 10 mice per group. 5T4-ADC, PF-384 and PTX were administered at clinically equivalent doses. All
procedures using mice were approved by the Pfizer Institutional Animal Care and Use Committee according to established guidelines. Time To Endpoint (time to no tumor or rate of tumor tripling) were used for comparison of drug-treated groups. T-test was used to compare individual tumor volumes as indicated in figure legends.

**Additional Materials and Methods are available in the Online Supplementary Information.**
Results

5T4-ADC or Auristatins Synergize with PI3K/mTOR Inhibitor In vitro

We performed a focused combinatorial screen of selected standard of care (SOC) agents or signaling inhibitors combined with auristatin-based drugs in a panel of breast, lung or ovarian cancer cells (unpublished data and Supplementary Table S1). Cell lines with previously characterized and clinically relevant 5T4 expression levels were also included in the analysis to enable direct comparison of the drug interactions with 5T4-ADC conjugate to the unconjugated auristatins (Supplementary Fig. S1, (7)). Through this screen we identified the dual PI3K/mTOR inhibitor PF-384 as showing most consistent interactions with microtubule-destabilizing agents or the 5T4-ADC across the panel of 16 cell lines (Fig. 1A). Overall, a similar pattern of synergistic or additive effects detected for 5T4-ADC was also observed for the cell permeable version of parental payload MMAF, MMAF-OMe or for a recently described auristatin analog PF-06380101 (Aur101)(5). Structurally unrelated microtubule modulators with diverse mechanisms of action (both stabilizer paclitaxel and destabilizer vinorelbine) also showed additive or synergistic relationships when combined with PF-384 in a smaller subset of cell lines, suggesting a shared mechanism of drug interaction that is directly related to inhibition of microtubules (Fig. 1A).

We also performed real-time monitoring of cell growth using the xCelligence system. Over the course of approximately 11 days, we observed that exposure of MDA-468 cells to 5T4-ADC/PF-384 and MMAF-OMe/PF-384 combinations reduced proliferation more than either of the individual agents alone (Fig. 1B and Supplementary Fig. S2A). Furthermore, when tested in 3D spheroid growth assays, significant enhancement of cytotoxicity was detected for the 5T4-ADC/PF-384 and MMAF-OMe/PF-384, compared with the single agent treatments (Fig. 1C and
Supplementary Fig. S2B). Similar observations were made for the lung cancer H-1975 spheroid model (data not shown). Thus, these results confirmed our findings above and provided additional insights on the time dependence of synergistic effects in vitro.

**Effect of 5T4-ADC/PF-384 on apoptosis, cell cycle and PI3K/mTOR markers**

To evaluate if synergistic growth inhibition induced by the 5T4-ADC and PF-384 combinations is due to apoptosis, we determined proapoptotic signal caspase 3/7 activation in H-1975 or MDA-468 cells. 5T4-ADC or PF-384 alone led to modest activation of caspase 3/7 in both cell lines, measured 24 hr post incubation (Fig. 1D). However, the combination treatment showed markedly enhanced induction of caspase 3/7. Similar results were obtained when the same cells were treated with MMAF-OMe/PF-384, suggesting that the induction of apoptosis in 5T4-ADC/PF-384 combination is mechanistically linked to the action of parental payload MMAF-OMe (Supplementary Fig. S2C). Immunoblot analysis for cleaved PARP further demonstrated the induction of apoptosis by the combination of 5T4-ADC/PF-384 in H-1975 (Fig. 1E). Collectively, these findings demonstrate that anti-proliferative effects observed with combinations could be accounted for, at least in part, by the enhanced apoptotic response mediated by caspase 3/7.

As auristatin-containing drugs have been described to impair cell cycle progression, we first asked if PF-384 can modify mitotic arrest when combined with 5T4-ADC in MDA-468 cells. As expected, 5T4-ADC alone markedly induced a significant accumulation of cells in mitosis as evidenced by the levels of phosphorylated histone H3 (P-H3) (Supplementary Fig. S3A). Interestingly, the addition of PF-384 substantially reduced ADC-mediated mitotic arrest. The same observations were made in H-1975 cells when PF-384 was combined with 5T4-ADC.
or free auristatins (Supplementary Fig. S3B and data not shown). Analysis of cell cycle in H-1975 cells showed that 5T4-ADC/PF-384 consistently reduced ADC-mediated G2/M arrest, with a modest increase in G1 stage compared to 5T4-ADC alone (Supplementary Fig. S3C). These results raised the possibility that combined treatment with 5T4-ADC/PF-384 may suppress the spindle assembly checkpoint function, leading to inappropriate transition out of mitosis. Aurora B inhibition has been shown to selectively relax the spindle checkpoint invoked by microtubule inhibitors (26, 27). We observed an increase of Aurora A, B and C phosphorylation by the 5T4-ADC, an effect blocked by the addition of 50-nM PF-384 (Supplementary Fig. S3D). Consequently, these results support the hypothesis that suboptimal Aurora activity may be responsible for accelerated mitotic exit and apoptosis in cells exposed to the combination of both drugs.

To examine changes in signaling after treatment with single agent PF-384 or 5T4-ADC, we initially used phospho-kinase antibody arrays. In the H-1975 lung tumor model treated with PF-384, we detected expected declines in P-AKT, P-S6K, P-GSK-3β, P-NOS and P-PRAS40 markers 6 or 16 hr following drug treatment (Supplementary Fig. S4A). Surprisingly, treatment with 5T4-ADC has resulted in the inhibition of P-AKT and P-GSK-3β 16 hrs post drug exposure (Supplementary Fig. S4B). To confirm these findings, we analyzed by immunoblot the downstream effectors of PI3K/mTOR pathways as well as markers for mitotic arrest and apoptosis. H-1975 (lower 5T4-expresser, L858R/T790M EGFR mutations) and MDA-468 (high 5T4-expresser, PTEN-/-) are cancer cell lines that are equally sensitive in vitro to the dual inhibitor of PI3K/mTOR or to auristatin derivative MMAF-OMe. We treated H-1975 or MDA-468 cells with the dual PI3K/mTOR inhibitor PF-384, 5T4-ADC, or a combination of the two. In agreement with the previous report (16), PF-384 alone effectively reduced phosphorylation of
downstream markers reflecting activation status of PI3K/mTOR pathway: P-AKT S308, P-AKT S473 and P-GSK-3α/β S21/9 24 hours after drug exposure (Fig. 1E and Supplementary Fig. S4C). In H-1975 cells, treatment with 50 μg/ml 5T4-ADC led to modest reduction in P-AKT S308, P-AKT S473, and GSK-3β (S9), as quantified by densitometry. In the same 24 hour experiment, 5T4-ADC/PF-384 treatment showed stronger decline in phosphorylation levels of PI3K/mTOR downstream effectors than each of the single agents, as confirmed through densitometric analysis (Fig. 1E). Increasing concentration of 5T4-ADC alone induced phosphorylation of H3, but the combination appeared nearly equivalent or even somewhat lower than 5T4-ADC alone, which is in good agreement with the mitotic index values determined by flow cytometry. Furthermore, we found that 5T4-ADC/PF-384 caused a greater induction of cleaved PARP than either 5T4-ADC or PF-384 alone. The potentiation of PF-384’s effect on downstream markers by 5T4-ADC appears to be cell line specific because experiments on MDA-468 showed no further reduction of P-AKT and P-GSK by the combination treatment as compared to PF-384 alone (Supplementary Fig. S4C). In MDA-468, we detected no suppression of PI3K/mTOR pathway markers with a single agent 5T4-ADC. These findings support the conclusions that enhanced cytotoxicity observed in 5T4-ADC/PF-384 combination is linked, at least in part, to the induction of caspase 3/7 and PARP–dependent apoptosis and correlates with the stronger suppression of PI3K/mTOR pathway biomarkers in H-1975 cells.

**Auristatin Agents Cooperate With PF-384 To Regulate Translation**

We hypothesized that previously uncharacterized effects of auristatins on additional intracellular targets may underlie the observed synergy with PF-384. Total proteomics and
phosphoproteomics were independently applied to evaluate changes in protein abundance and phosphorylation in response to auristatin-based agents in MDA-468, a cell line that had no demonstrable changes in phosphorylation of AKT or GSK upon administration of 5T4-ADC.

Analysis of protein interaction networks for all significantly changed proteins in total proteome perturbed with 0.5 nM auristatin showed several clusters of related functional classes of proteins, with a highly distinct group containing components of mRNA translation and mRNA biogenesis (Fig. 2A, Supplementary Fig. S5A). Specifically, we detected remarkable overrepresentation of mRNA translation factors in the down-regulated protein set and of ribosomal proteins in the up-regulated group after a 24 hr cell exposure to either of the two doses of MMAF-Ome (0.5 and 5 nM) (Supplementary Fig. S5B, S5C and Supplementary Table S2A). Label-free phosphoproteomics of cells treated with the MMAF-Ome or 5T4-ADC for 6 hr demonstrated unanticipated enrichment of “translation factors”, “mRNA processing” and “mRNA splicing” for both, MMAF-Ome and 5T4-ADC in the list of down-regulated phosphopeptides (Supplementary Fig S5D, S5E and Supplementary Table S2B). Consistent with the common mechanism of action between the ADC and free unconjugated payload, there was a substantial overlap in differentially expressed phosphopeptides between MMAF-Ome and 5T4-ADC, with the overrepresentation of translation or mRNA processing-related components in the shared datasets (Supplementary Fig. S5D). Thus, our results raise the intriguing possibility that protein synthesis is one of the convergence points for cellular action of MMAF-Ome or 5T4-ADC. This notion, together with the well-known role for PI3K/mTOR signaling pathway in the regulation of protein synthesis (28, 29), supports the hypothesis that cellular synergy of 5T4-ADC/PF-384 or MMAF-Ome/PF-384 might be at least partially attributed to cooperative inactivation of translation.
To substantiate the above findings, we focused on the effects of 5T4-ADC/PF-384 combination on steady-state level and phosphorylation status of key translation factors involved in PI3K/mTOR pathway. Single agent 5T4-ADC showed modest cell type-specific effects, each modulating expression and/or phosphorylation of a select subset of proteins to a different extent (Fig 2B). In both cell lines, treatment with 5T4-ADC decreased levels of eIF4G1, eIF4G2, eIF4B, eIF3A, but upregulated P-eIF2α and P-eEF2. More importantly, combination treatments caused cooperative changes in a distinct set of translational regulators. For example, in MDA-468, a cell model that was used for proteome-wide analysis, 5T4-ADC/PF-384 combination led to a stronger decline in eIF4G2, eIF4B, eIF2α, eIF3A levels, which coincided with greater induction of P-eIF2α and P-eEF2 than for each agent alone (Fig 2B). In the H-1975 model, the same drug combination caused decreases in the expression levels of P-eIF4G1, P-eIF4B, eIF4B, P-4E-BP1, 4E-BP1 with a concomitant increase in P-eIF2α and P-eEF2. To test whether these observations also translate into functional impairment of general protein synthesis, we monitored the activity of firefly luciferase in MDA-468 cells stably transduced with a cap-dependent monocistronic reporter. Addition of PF-384 or 5T4-ADC significantly suppressed production of luciferase by approximately 40-50% after 16 or 24 hr incubation with drugs, whereas treatment with a positive control, protein synthesis inhibitor cycloheximide (CHX), fully reduced luciferase production in this system. Treatment with 5T4-ADC/PF-384 led to a stronger decline in luciferase activity when compared with either single agent alone (Fig. 2C). No reduction of luciferase transcription was observed in this experiment in response to drug exposure as measured by RT-PCR (data not shown). Overall, our results suggest that combining auristatin-based drugs with inhibitors of PI3K/mTOR pathway can lead to a specific reprogramming of
translational factor repertoire at the level of expression and/or phosphorylation, which causes suppression of protein synthesis.

**In vivo combination therapy with 5T4-ADC and PF-384**

To investigate whether cooperative action of 5T4-ADC/PF-384 *in vitro* could be observed in the *in vivo* setting, we tested the efficacy of the respective single agents and of the combination in two previously characterized tumor xenograft models with broad range of 5T4 expression levels as shown by flow cytometry and IHC staining (Supplementary Fig. S1B and (7)). Treatment of animals bearing MDA-468 breast cancer xenografts with 2 mg/kg 5T4-ADC caused initial robust tumor suppression followed by stasis, whereas PF-384 at 7.5 mg/kg showed a very minor inhibition of tumor growth over the vehicle-treated arm (Fig. 3A). In contrast, concurrent administration of both drugs led to more complete tumor regressions clearly observed in all tumors treated by the end of the study. Subsequent time-to-endpoint (TTE) analysis of time to no tumor (tumor regression) showed that a much shorter time was needed to achieve complete tumor regressions in the combination arm versus 5T4-ADC alone (*p* < 0.0001 by the log-rank test), with all animals in the 5T4-ADC/PF-384 group becoming tumor-free by the day 36 (Fig. 3B). In the H-1975 lung cancer model, treatment with 3 mg/kg 5T4-ADC resulted in tumor stasis followed by regrowth of tumors, while 7.5 mg/kg PF-384 elicited only nominal antitumor activity (Fig. 3C). The 5T4-ADC/PF-384 combination resulted in a more complete, but still unsustained suppression of tumor growth. The percentage of animals with less than 3-fold increase in tumor volume was used as survival endpoint for the analysis of H-1975 model. TTE analysis indicated a statistically significant delay in tumor tripling rate for the combination.
group compared to 5T4-ADC (3 mg/kg; \( p = 0.0356 \), Log-rank test) or PF-384 alone (7.5 mg/kg, \( p < 0.0001 \), Log-rank test, Fig. 3D).

**Combination of 5T4-ADC with taxanes**

The initial drug interaction screen also identified PTX as an agent that potentiated MMAF-OMe- or 5T4-ADC growth inhibitory effects. The combination results varied from synergism to additivity in most of the cell models tested as measured by the CI index (Fig. 4A). This effect was not unique to PTX, as docetaxel, a structurally similar taxane, also showed favorable interactions with MMAF-OMe or 5T4-ADC. Moreover, when MMAF-OMe or 5T4-ADC was substituted for another auristatin analog Aur101, or an unrelated microtubule-depolymerizing agent vinorelbine, we also observed potentiation of their cytotoxicity by PTX. According to a high-resolution structural data obtained for dolastatin-10 (30) or for the new auristatin analog bound to tubulin (5), auristatins bind at a site adjacent to the vinca binding site at the interface of two tubulin molecules and in close proximity to the \( \beta \)-tubulin nucleotide exchange (Fig. 4A, *Right*). We examined additional cellular changes after co-treatment with PTX. Modest enhancement of cytotoxicity in a 3D spheroid assay and stronger induction of caspase 3/7 was observed in MDA-468 cells treated with 5T4-ADC plus PTX for 48 hrs compared to the single drug controls (Figs 4B and 4C). A potential explanation for the cooperative action between 5T4-ADC and PTX includes modulation of cell cycle progression and altered microtubule dynamics. As expected, an M-phase specific marker P-H3 was markedly induced in MDA-468 cells treated with PTX or 5T4-ADC. The co-treatment with both drugs slightly enhanced the mean mitotic increase relative to single agents, but this trend did not reach statistical significance (Supplementary Fig. S6A). Furthermore, we observed enhanced PARP cleavage and increase in
levels of P-H3 in MDA-468 or H-1975 cells treated with 5T4-ADC/PTX combination versus either single drug (Fig. 4D). Interestingly, fluorescent microscopy with an anti-tubulin antibody demonstrated a significant collapse of microtubule network around the nucleus and formation of the microtubule aggregates with 5T4-ADC/PTX combination in MDA-468 cells (Supplementary Fig. S6B). Unlike the combination’s effect, the 5T4-ADC alone treated cells showed disintegration of microtubule bundles with more intense staining at cell periphery and lesser cytoplasmic volume. Collectively, these findings demonstrate that anti-proliferative effects observed with 5T4-ADC/PTX could be accounted for, at least in part, by the enhanced apoptotic response mediated by caspase 3/7 and PARP with parallel induction of P-H3 and more pronounced loss of microtubule integrity.

Given the favorable interactions observed between 5T4-ADC and taxanes in vitro, we also evaluated the potential antitumor activity of this combination in vivo. MDA-468 xenografts were tested with concurrent combinations of 5T4-ADC (2 mg/kg) plus PTX (10 or 22.5 mg/kg) (Figs. 5 A-D). PTX monotherapy had no pronounced effect on tumor growth at 10 mg/kg, but resulted in strong antitumor activity at the higher dose of 22.5 mg/kg (Fig. 5A, C). Single drug treatment with 2 mg/kg 5T4-ADC in this model led to sustained but incomplete tumor regression followed by the stasis. However, coadministration of 5T4-ADC and PTX at two different doses resulted in profound and lasting tumor regression for the duration of the study. Importantly, TTE analysis of these combinations revealed significantly shorter time needed to achieve complete tumor regressions for the combination arms compared to single drugs alone (Fig. 5B, D). In H-1975 human lung cancer xenograft model, PTX treatment at 10 mg/kg resulted in tumor growth delay (Fig. 5E). 5T4-ADC at 3 mg/kg inhibited the growth of xenografts, with tumor regrowth evident 2 weeks after treatment was stopped. In contrast, the dual treatment with 5T4-ADC/PTX
produced marked enhancement in anti-tumor activity compared to monotherapy treatments. Moreover, log-rank tests showed statistically significant delay in tumor tripling rate for the combination group compared to 5T4-ADC or PTX alone treatment arms (Fig. 5F). In summary, these data suggest that PTX, when used at clinically achieved exposures, can strongly enhance anti-tumor efficacy of 5T4-ADC in preclinical models of human lung and breast cancer.

Discussion

Optimizing the efficacy of ADCs by systematic non-clinical assessment of combinations remains an important objective for ADC development. We hypothesized that major synergistic effects for the antibody-auristatin conjugates may be mediated by the pharmacological action of payload itself and therefore searched for the common chemotherapeutic agents or signaling inhibitors that could potentiate either free- or conjugated auristatins such as 5T4-ADC. Here we describe novel and previously uncharacterized potentiation of auristatin-based agents by PF-384 or taxanes in vitro that translates to enhanced anti-tumor efficacy in tumor xenograft models. The combination of MMAF-Ome or 5T4-ADC and PF-384 has resulted in consistently synergistic drug effect in tumor cell lines of lung, breast and ovarian cancer origin, the three putative tumor types which show broad 5T4 expression (7). Analysis of the in vitro cytotoxicity data for the cell line panel used in this study revealed that neither common mutations in these cell lines nor their tissue lineage or sensitivity of the individual drugs alone could be used to predict synergistic responses to the combinations involving auristatins and other drugs. The favorable pharmacological interactions were also observed when different microtubule-targeting agents paclitaxel (polymerizing) or vinorelbine (depolymerizing, unpublished observation) were
combined with PF-384, suggesting that microtubule damage is a global signal that can be potentiated by the suppression of PI3K/mTOR signaling. Surprisingly, we also found reproducible but cell- and drug-dependent inhibition of AKT and/or GSK3 phosphorylation in response to single-agent 5T4-ADC. This finding is unexpected but in agreement with the report by Asnaghi and coworkers who demonstrated inhibitory effects of nocodazole on phosphorylation of mTOR at Ser 2448 (31). We however could not consistently detect synergistic suppression of PI3K/mTOR pathway markers in MDA-468 cells. Unlike H-1975, an MDA-468, a PTEN-/- breast cancer model is more sensitive to each single agent alone, which may make the detection of cooperative effects on downstream pathways technically difficult.

We also applied a systems-wide approach to explore the additional cellular action of auristatins that could help explain molecular mechanisms behind the synergistic interactions with PF-384. Both total proteomics with MMAF-OMe and phosphoproteomics performed with 5T4-ADC and MMAF-OMe uncovered mRNA translation as one of the predominantly affected processes. These findings were further corroborated by western blot analysis of selected translation initiation or elongation components. More proteins were modulated by MMAF-Ome, 5T4-ADC or PF-384 alone, with only some showing cooperative effects by combined treatment with 5T4-ADC/PF-384 or MMAF-OMe / PF-384. This comports with other large-scale phosphoproteomic studies that identified a number of initiation factors differentially phosphorylated in response to nocodazole treatment (32, 33). Whereas targeting of translational components by auristatin-based agents is a novel finding, the effects of PF-384 are quite expected, given the known role of PI3K and mTOR kinases in the control of protein synthesis. One anticipated consequence of ADC-mediated modulation of protein synthesis machinery is a decline in global translation rates. Stronger reduction of cap-dependent synthesis of luciferase
reporter by the 5T4-ADC/PF-384 combination relative to single-agent drugs is consistent with upregulation of P-eIF2α (S51) and P-eEF2 (T56), changes that are suggestive of a slowdown in translation. Thus, cooperative suppression of protein synthesis can, at least in part be linked to the observed cellular synergy with PF-384. Notably, structurally unrelated and highly specific mTOR inhibitor WYE-132, which is known to disrupt the cap-dependent mRNA translation and inhibit global protein synthesis, also showed synergy when combined with auristatin derivative in vitro (Supplementary Table S3). This implies that reducing cap-dependent translation by targeting mTOR kinase activity alone may be sufficient to enhance therapeutic effects of auristatin-based agents.

Given the highly specific effects of MMAF-OMe and 5T4-ADC on microtubules, how these agents perturb mRNA translation and how PF-384 can potentiate this mechanism? One potential explanation is based on the substantial evidence of interactions between tubulin cytoskeletal components and ribosomal proteins, translation initiation factors and various mRNPs. In addition, many mRNAs encoding mitotic regulators or translational components are known to localize to mitotic spindles (34-40). This and the evidence that ongoing translation is maintained throughout the cell cycle, without substantial decline during mitosis (35) is consistent with the notion that tubulin-localized protein synthesis maybe especially important for the efficient progression through mitosis. Hence, we propose that entire class of auristatin-containing agents, including ADCs can disrupt the tubulin-bound pools of translational components, thereby modulating their abundance. We speculate that the combined action of ADC and PF-384 disrupts protein synthesis during mitotic transition or collectively impacts translation of the key mRNAs required for the survival during mitotically arrested state. Furthermore, it is also possible that both drug classes could affect mRNA translation in different
stages of cell-cycle. Interestingly, co-treatment with PF-384 impairs Aurora kinase phosphorylation in our experiments, a phenotype that is generally consistent with compromised spindle checkpoint and decreased mitotic index in these cells. These data are also in agreement with what has been previously described for combination of docetaxel and another PI3K inhibitor GDC-0941, where decreased time of mitotic arrest was mechanistically linked to the induction of apoptosis in synchronized cells (41). Our results provide one potential scenario to explain the synergistic activity and it is likely that multiple mechanisms may determine favorable pharmacological outcomes in tumor cells.

Remarkably, the combination therapy with 5T4-ADC/PF-384 in vivo significantly improved anti-tumor activity and reduced tumor volumes in models of breast and lung cancer as compared to the effects of single drug treatment alone. Other important preclinical work demonstrated that T-DM1 plus the pan-PI3K inhibitor GDC-0941 or plus dual PI3K/mTOR combinations resulted in the enhanced anti-tumor activity both in vitro and in trastuzumab-resistant or in PIK3CA mutant breast cancer xenograft models in vivo (13). The T-DM1 plus GDC-0941 was tested in a 3+3 design dose-escalation phase Ib study in patients with advanced HER2-positive metastatic breast cancer. Results have been reported only on 13 patients, with dose-liming toxicities (DLTs) of a grade 4 thrombocytopenia and grade 3 fatigue observed in two initial cohorts. The combination regimen was better tolerated in a third cohort which enrolled at a reduced dose of T-DM1 (3.0 mg/kg) and GDC-0941 (100 mg) (42). It remains to be seen if combining T-DM1 with PI3K inhibitors generates meaningful clinical activity in these patients.

Several lines of evidence illustrate an important therapeutic potential for another promising combination between the 5T4-ADC and a taxane, PTX. Enhanced anti-tumor activity
of 5T4-ADC plus PTX is intriguing but not counterintuitive. Whereas both agents act on the microtubules, MMAF-OMe binds to a distinct site than the taxane binding site, in a manner similar to the vinca alkaloids and thus may affect additional tubulin-dependent functions in a PTX-independent manner. This may lead to therapeutic synergy when combined with PTX. There is considerable interest in the ADC field in testing the clinical activity and safety of the conjugates with other cytotoxic anti-mitotic agents, such as taxanes. This hypothesis is currently under investigation in clinical trial evaluating combination therapy of T-DM1 plus docetaxel in early stage HER+ breast cancer (43) and in a phase III study comparing brentuximab vedotin plus AVD vs ABVD (doxorubicin, bleomycin, vinblastine, and dacarbazine) alone (44).

In conclusion, our report provides strong preclinical framework and the rationale for combination therapy of 5T4-ADC with taxanes or 5T4-ADC with PF-384 in clinical trials for the treatment of lung, breast, or ovarian cancer. The dual targeting approach presented here, with both an auristatin-based agent and PI3K/mTOR pathway inhibitor or taxanes, could serve as an important model for enhancing antitumor activity of other auristatin-based ADCs and overcoming potential drug resistance in the clinic.
Authors' Contributions

Conception and design: Boris Shor, Puja Sapra.

Development of methodology: Boris Shor, Maureen Dougher, Jennifer Kahler, Jane Xu, Michelle Mack

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Boris Shor, Puja Sapra

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Boris Shor, Maureen Dougher, Jennifer Kahler, Jane Xu, Michelle Mack, Puja Sapra

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Boris Shor, Maureen Dougher, Jennifer Kahler, Jane Xu, Michelle Mack

Writing, review, and/or revision of the manuscript: Boris Shor, Puja Sapra

Study supervision: Boris Shor, Puja Sapra.

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References


Figure Legends

Figure 1. Effects of auristatin-based agents combined with PF-384. A, Dot plot summary showing the range of CI index values in a panel of cancer cell lines obtained following analysis of drug combinations with PF-384 or with mTOR-specific inhibitor. CI indexes were determined using Chou Talalay method as described in Materials and Methods and are presented at the ED50 level for each of the combinations. Results are the average of at least three independent experiments. The CI has been interpreted as follows: very strong synergy (<0.1), strong synergy (0.1 to 0.3), synergism (0.3 to 0.7), moderate synergism (0.7 to 0.85), slight synergism (0.85 to 0.9), nearly additive (0.9 to 1.1), slight antagonism (1.1 to 1.2) and moderate antagonism (1.2 to 1.45). Dashed lines are at CI values of 1.1 and 0.7. PTX, paclitaxel; VINO, vinorelbine; Aur101, auristatin-101. B, Dynamic monitoring of cell growth in MDA-468 cells with the xCELLigence System. B, Left, concentration- and time-dependent cytotoxic effects of 5T4-ADC, PF-384 or combination in MDA-468 cells. One representative experiment of three is shown. B, Right, Impedance index values are presented for the 8-day time point and show statistically significant enhancement of cell growth inhibition for the 5T4-ADC plus PF-384 combination. (***p < 0.001; Student’s t test). The means and SEM are shown. C, MDA-468 cells grown as 3D spheroids matrigel were treated with increasing doses of 5T4-ADC, a fixed dose of PF-384 (10 nM) or a combination of both drugs for 7 days. Spheroid viability was measured as described in Materials and Methods. Plotted is the percentage of growth relative to the respective untreated control. Values are means ± SEM. Dashed line indicates % viability for the fixed dose of PF-384. B, Right, Histogram plots of the selected data points at the indicated drug concentrations. Asterisks represent significant differences from 5T4-ADC+PF-384 (**p < 0.01, Student’s t test). D, Enhanced induction of caspase 3/7 in MDA-468 (Left) or H-1975 (Right).
(Right) cells treated with 5T4-ADC (10 μg/ml) plus PF-384 (1 μM) for 24 hrs. Fold induction in caspase 3/7 activity was determined as described in Materials and Methods. Means and SEMs of triplicate experiments are shown. *p < 0.05, **p < 0.01, ***p < 0.001. Asterisks show statistically significant differences between each of the single drugs alone and a combination; student’s t test. E, H-1975 cells were treated with indicated concentrations of 5T4-ADC and PF-384, or concurrently with the combination of both for 24 hr. Cell lysates were subjected to immunoblotting analysis with antibodies to P-AKT, AKT, P-GSK, GSK, P-H3, cPARP or with antibodies to GAPDH as loading control. E (Right) Densitometric analyses of (E) was performed using ImageJ, as described in Supplementary Materials and Methods. Densitometric analyses of data shows protein levels of P-AKT (S473, T308), P-GSK3β (S9).

Figure 2. Cooperative suppression of protein translation by ADC/PF-384 combination. A, STRING network analysis of all significantly changed proteins in a total proteomics experiment for MDA-48 cells treated with 0.5 nM MMAF-Ome. Major cluster of interacting proteins includes the translation factors, ribosomal proteins and ribonucleoproteins that form a densely connected module, which is denoted by red rectangle. Only connected nodes are shown for simplicity. STRING network analysis and visualization was performed using the online STRING database 9.1 (45). B, Total protein abundance and phosphorylation status of selected proteins involved in mRNA translation. MDA-468 (left) or H-1975 (right) cells were treated with the 5T4-ADC, PF-384 or combinations of both drugs for 24 hours at the indicated concentrations. Total protein abundance or phosphorylation levels were measured with antibodies indicated and as described in Materials and Methods. C, Effect of auristatin-based agents alone or in combination with PF-384 on cap-dependent synthesis of luciferase reporter.
MDA-468 cells stably transduced with monocistronic luciferase reporter were used to assay inhibition of cap-dependent translation as described in Materials and Methods. Cells were treated with individual drugs or with combinations for 24 hours. The concentrations of drugs were, PF-384 50 nM, 5T4-ADC 10 μg/ml, PTX (paclitaxel) 10 nM, CHX (cycloheximide) 30 μg/ml. 5T4-ADC/PF-384: 5T4-ADC 10 μg/ml and PF-384 50 nM. Results are mean±s.e. of biological triplicates from a single experiment representative of two. 5T4-ADC and PF-384 were significantly different compared with vehicle-treated control by 2-tailed Student's t-test. 5T4-ADC/PF-384 combination was significantly different compared with each of the single drug-controls by 2-tailed Student's t-test.

**Figure 3.** The 5T4-ADC plus PF-384 combination treatment lead to enhanced therapeutic effects in breast and lung cancer models *in vivo*. A, B Mice bearing subcutaneous MDA-468 human breast tumor xenografts were treated with vehicle, 5T4-ADC (i.v. 2 mg/kg, Q4D), PF-384 (i.v. 7.5 mg/kg, Q4D) or a combination. A, Tumor growth curves. Tumor volume was determined at the indicated times after the onset of treatment. Points, mean of values from 10 mice/group; bars, SE. B, Time to Endpoint (TTE) plots for the treatment groups in (A) show change in percent of animals with tumors over the time. Endpoint is defined as the time elapsed for animal to become tumor-free. TTE analysis demonstrates significantly enhanced rate of tumor regressions with combination of 5T4-ADC plus PF-384 compared to the single agent activity of 5T4-ADC (p < 0.0001, log-rank Mantel-Cox test). PF-384 did not elicit regressions in this experiment. C, D, Mice bearing subcutaneous H-1975 human lung tumor xenografts were treated with 5T4-ADC (i.v. 3 mg/kg, Q4D), PF-384 (i.v. 7.5 mg/kg, Q4D) or a combination. C, Tumor growth curves. Tumor volume was determined at the indicated times...
after the onset of treatment. Points, mean of values from 10 mice/group; bars, SE. TTE plots for
the treatment groups in (C) show the percentage of animals with less than 3-fold increase in
tumor volume over time. Endpoint is defined as the time at which tumor volume has tripled.
TTE analysis of data demonstrates significant delay at rate of tumor tripling for the 5T4-ADC
plus PF-384 combination compared to the single agent activity of 5T4-ADC (p = 0.0356, log-
rank test) or PF-384 (p < 0.0001 log-rank test) alone. To minimize fluctuations in the tumor
growth curve plots and facilitate interpretation of the data, the mean tumor volume for each
group was plotted until >10% of the mice in the group were sacrificed.

**Figure 4.** Effects of auristatin-based agents combined with taxanes. A, Summary of
Combination Index (CI) values in a panel of cancer cell lines. Dot plot showing the range of CI
index values obtained following analysis of drug combinations with microtubule inhibitors. CI
indexes were determined using Chou Talalay method as described in Materials and Methods and
are presented at the ED50 level for each of the combinations. Results are the average of at least
three independent experiments. The CI has been interpreted as in Fig 1A. Dashed lines are at CI
values of 1.1 and 0.7. B, Combination of 5T4-ADC or MMAF-OMe with PTX leads to stronger
suppression of cell growth in 3D culture. MDA-468 cells were treated with increasing doses of
5T4-ADC, fixed dose of PTX (1 nM) or a combination of both drugs for 7 days. Spheroid
viability was measured as described in Materials and Methods. Plotted is the percentage of
growth relative to the respective untreated control. Dashed line indicates % viability for the
fixed dose of MMAF-OMe. Right, Histogram plots of the selected data points at the indicated
drug concentrations. C, Induction of caspase 3/7 activity by the combination of 5T4-ADC with
PTX in MDA_468 cells. Left, Cells treated with 5T4-ADC (10 μg/ml) plus PF-384 (1 μM);
right, cells treated with MMAF-OMe (0.22 nM) plus PTX (6 nM) (D) for 48 hr. Fold induction
in caspase 3/7 activity was determined as described in Materials and Methods. Means and SEMs of triplicate experiments are shown. *p < 0.05, **p < 0.01, ***p < 0.001. Asterisks show statistically significant differences between each of the single drugs alone and a combination; student’s t test. D, Effect of single agent 5T4-ADC and combinations with PTX on apoptosis and mitotic marker modulation. MDA-468 (top) or H-1975 (bottom) cells were treated with indicated concentrations of 5T4-ADC and PTX, or concurrently with the combination of both for 24 hr. Cell lysates were subjected to immunoblotting analysis with antibodies to cPARP, P-H3(S10), or with antibodies to GAPDH as loading control.

**Figure 5.** The 5T4-ADC and PTX combination treatment leads to enhanced therapeutic effects in breast and lung cancer models *in vivo*. A, C Mice bearing subcutaneous MDA-468 human breast tumor xenografts were treated with 5T4-ADC (i.v. 2 mg/kg, Q4D), PTX (p.o. 10 mg/kg, Q4D) or a combination. A, Tumor growth curves. Tumor volume was determined at the indicated times after the onset of treatment. Points, mean of values from 10 mice/group; bars, SE. B, Time to Endpoint (TTE) plots for the treatment groups in (A) show change in percent of animals with tumors over the time. Endpoint is defined as the time elapsed for animal to become tumor-free. TTE analysis of data demonstrates significantly faster rate of complete tumor regressions achieved with the combination of 5T4-ADC and PTX compared to the single agent activity of 5T4-ADC (p = 0.0071, log-rank test) or PTX (p=0.01, log-rank test). C, Similar to (A), but PTX was used at a dose of 22.5 mg/kg (p.o. Q4D). D, TTE analysis of data from (C) shows significantly faster rate of complete tumor regressions achieved with the combination of 5T4-ADC plus PTX compared to the single agent activity of 5T4-ADC (p = 0.00821, log-rank test) or PTX, which has not produced any tumor regressions at this dose. E, Mice bearing
subcutaneous H-1975 tumors were treated with 5T4-ADC (i.v. 3 mg/kg, Q4D), PTX (p.o. 10 mg/kg, Q4D) or a combination. 5T4-ADC combined with PTX is more efficacious than treatment with single agents. F, TTE analysis of data performed similarly to Fig. 3B, demonstrates significant delay of rate of tumor tripling for the 5T4-ADC plus PTX combination compared to the single agent activity of 5T4-ADC (p < 0.0001, log-rank test) or PTX (p = 0.0001 log-rank test) alone.
**A. Combinations with PF-384**

- MDA-468 Spheroid Growth, 7 Days
- MDA-468, Spheroid Growth
- H-1975, 24 h

**B. MDA-468, 8 Days**

- Control
- PF-384 (50 nM)
- 5T4ADC (1 µg/ml)
- 5T4ADC+PF384

**C. MDA-468 Spheroid Growth, 7 Days**

- 5T4-ADC (µg/ml)
- PF-384 (10 nM)

**D. MDA-468, 24h**

- Fold Induction (Caspase 3/7)

**E. H-1975, 24h**

- 5T4-ADC, µg/ml
- PF-384, nM

**Fig. 1**
Fig. 2

A.

B.

MDA-468

- - - 1     10 1      1 10 10 5T4-ADC, µg/ml
- 5 50 - - 5 50 5 50 PF-384, nM

P-eIF4G1 (S1108)
eIF4G1
eIF4G2
P-eIF4B (S422)
eIF4B
P-eIF2α (S51)
eIF2α
eIF3A
P-eEF2 (T56)
eEF2
P-4E-BP1 (T37/46)
4E-BP1
GAPDH

H-1975

- 10 50 - - 10 50 50 50 5T4-ADC µg/ml
- - 5 50 5 50 5 50 PF-384, nM

P-eIF4G1 (S1108)
eIF4G1
eIF4G2
P-eIF4B (S422)
eIF4B
P-eIF2α (S51)
eIF2α
eIF3A
P-eEF2 (T56)
eEF2
P-4E-BP1 (T37/46)
4E-BP1
GAPDH

C.

MDA-468-Luc

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Combination with Taxanes

Combinations with Taxanes

MDA-468 Spheroid Growth, 7 Days

MDA-468, 48h

Fold Induction (Caspase 3/7)

Auristatin
Taxol
GDP
GTP

Fig. 4
Clinical Cancer Research

Enhanced Anti-Tumor Activity Of an anti-5T4 Antibody-Drug Conjugate in Combination with PI3K/mTOR inhibitors or Taxanes

Boris Shor, Jennifer Kahler, Maureen Dougher, et al.

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